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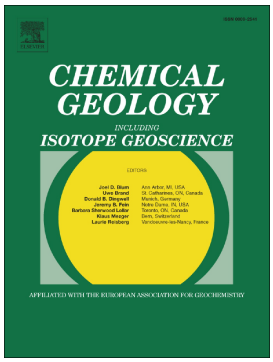

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Three wood isotopic reference materials for δ^2H and δ^{13}C measurements of plant methoxy groups

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Highlights

- Three wood isotopic reference materials were prepared to serve as long-term reference materials for plant methoxy groups
- Methoxy groups from homogenized wood samples were quantitatively converted to iodomethane

- δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values of generated iodomethane were calibrated against international reference substances
- δ^2H_{OCH3} values of the three wood samples span a relatively wide range (~-280 to -190 mUr)
- The three investigated wood materials HUBG3-5 are ideally suited for long-term usage and inter-laboratory comparison

Abstract

Methoxy groups (OCH_3) of plants show specific stable carbon and hydrogen isotope patterns that are used for applications in biogeochemical, atmospheric, paleoclimatic and food research. The method of choice for determining stable hydrogen and carbon isotope values of methoxy groups (δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values) is the conversion to gaseous iodomethane (CH_3I) and subsequent measurement by stable isotope ratio mass spectrometry. However, comparative measurements particularly for stable hydrogen isotopes of plant methoxy groups are limited due to the lack of suitable reference materials. We have prepared three batches of powdered wood samples (birch HUBG3, beech HUBG4, and tineo HUBG5) collected from different geographical locations to serve as long-term reference materials for normalization of δ^2H_{OCH3} but also $\delta^{13}C_{OCH3}$ values. Methoxy contents of the three wood samples range between 4.7 and 5.4%. Methoxy groups from subsamples of the three homogenized wood samples were quantitatively converted to CH_3I and δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values of this CH_3I were calibrated against international reference substances by high-temperature conversion- and elemental analyzer isotope ratio mass spectrometry. The δ^2H_{OCH} and $\delta^{13}C_{OCH3}$ values of HUBG3 and HUBG4 at 1σ uncertainty calibrated to the VSMOW and VPDB isotopic δ-scale, respectively are -272.9 ± 1.5 mUr and -29.40 ± 0.13 mUr; and -239.1 ± 1.4 mUr and -30.17 ± 0.13 mUr, respectively. In addition, the calibrated δ^2H_{OCH} value of HUBG5 is -191.7 ± 0.8 mUr. Whilst the δ^2H_{OCH3} values of the three wood samples span a relatively wide range (~-280 to -190 mUr) suitable for normalization of δ^2H_{OCH3} values of most plant samples that have been reported so far, $\delta^{13}C_{OCH3}$ values are restricted to a composition (~-30 mUr) typical of wood methoxy groups. We suggest that the three investigated wood materials HUBG3-5

are ideally suited for long-term usage, inter-laboratory comparison, and together with the two recently reported methyl sulfate salts (HUBG1 and HUBG2) complete a new set of solid methoxy reference materials that cover almost the full range of plant methoxy groups reported so far. Therefore, we recommend replacing liquid CH_3I and instead use the solid reference materials set HUBG1-5 for normalizing δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values to the respective δ-scales.

Keywords

Plant methoxy groups; solid reference materials; stable hydrogen isotopes; stable carbon isotopes; stable isotope mass spectrometry

1. Introduction

Methoxy groups (OCH_3) of lignin and pectin constitute a significant fraction of the biospheric C_1 methyl (CH_3) pool of plant origin accounting for ~4-6% of wood dry mass (Galbally and Kirstine, 2002; Keppler et al., 2004) and ~1-3% of leaf dry mass (McRoberts et al., 2015). In the past 15 years several studies have shown that methoxy groups of plants have both distinct stable hydrogen (δ^2H_{OCH3}) and carbon ($\delta^{13}C_{OCH3}$) isotope values (Keppler et al., 2004) with reported δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values ranging from -149 mUr (Greule et al., 2010) to -405 mUr (Anhäuser et al., 2018) and -7.1 mUr (Greule et al., 2010) to -77.2 mUr (Keppler et al., 2004), respectively. These specific δ^2H and $\delta^{13}C$ isotope patterns have considerable potential for application as tools for investigations in biogeochemical, atmospheric, paleoclimatic and food research (Greule et al., 2019). For a more detailed overview of its applications in environmental research we refer to previous studies by (Anhäuser et al., 2014; Feakins et al., 2013a; Greule et al., 2012, 2010; Hepp et al., 2017; Keppler et al., 2004; Lee et al., 2019). Please also note that we follow the suggestion by Brand and Coplen, 2012 and express isotope delta values in milli-Urey [mUr] (after Urey, 1948) instead of per mil [‰] (1 mUr = 1 ‰).

Most measurements of site-specific isotopic abundances of organic molecules are accomplished by nuclear magnetic resonance (NMR) spectroscopy, which requires relatively large masses of chemically pure samples. This complicates the application of this technique to most natural samples. By combining a long-established technique for the derivatization of plant methoxy groups to gaseous iodomethane (CH_3I) using hydriodic acid (Zeisel, 1885) with conventional methods for continuous flow isotope ratio mass spectrometry (CF-IRMS), it is possible to determine the ^{13}C and ^{2}H isotope compositions of plant methoxy groups with high precision and no apparent isotopic fractionation on milligram quantities of bulk tissue (Greule et al., 2009, 2008).

Stable isotope analyses require reference materials to normalize stable isotope ratios on the respective δ-scale (Brand et al., 2014; Carter and Barwick, 2011; Meier-Augenstein and Schimmelmann, 2019; Werner and Brand, 2001) Highest accuracy is achieved when workflows adhere to the principle of "identical treatment of sample and reference material" (IT) in preparation and analysis, which requires that samples are chemically analogous to standards of known isotopic composition (Meier-Augenstein and Schimmelmann, 2019; Schimmelmann et al., 2016; Werner and Brand, 2001) When selecting reference materials for IRMS analyses further criteria have to be considered as outlined in detail by several previous studies, such as isotopic homogeneity, unchanging stable isotopic composition over time, chemical similarity to the samples, easy preparation, storage and handling as well as no health risk (Brand et al., 2014; Carter and Barwick, 2011; Meier-Augenstein and Schimmelmann, 2019; Schimmelmann et al., 2016; Werner and Brand, 2001) Most published stable isotope values of methoxy groups analyzed by IRMS were normalized to the respective δ-scale using liquid CH_3I as the reference material. However, this practice violates the above principles because liquid CH_3I, unlike the sample, is typically not heated at 130°C with HI for derivatization (Greule et al., 2019). Moreover, $\delta^{2}H$ and $\delta^{13}C$ values of commercially available CH_3I have restricted ranges ($\delta^{2}H$: -66 to -179 mUr; $\delta^{13}C$: -46 to -70 mUr) (Feakins et al., 2013b; Greule et al., 2019; Keppler et al., 2007) that do not bracket typical $\delta^{2}H_{OCH3}$ and $\delta^{13}C_{OCH3}$ values of plant methoxy groups.

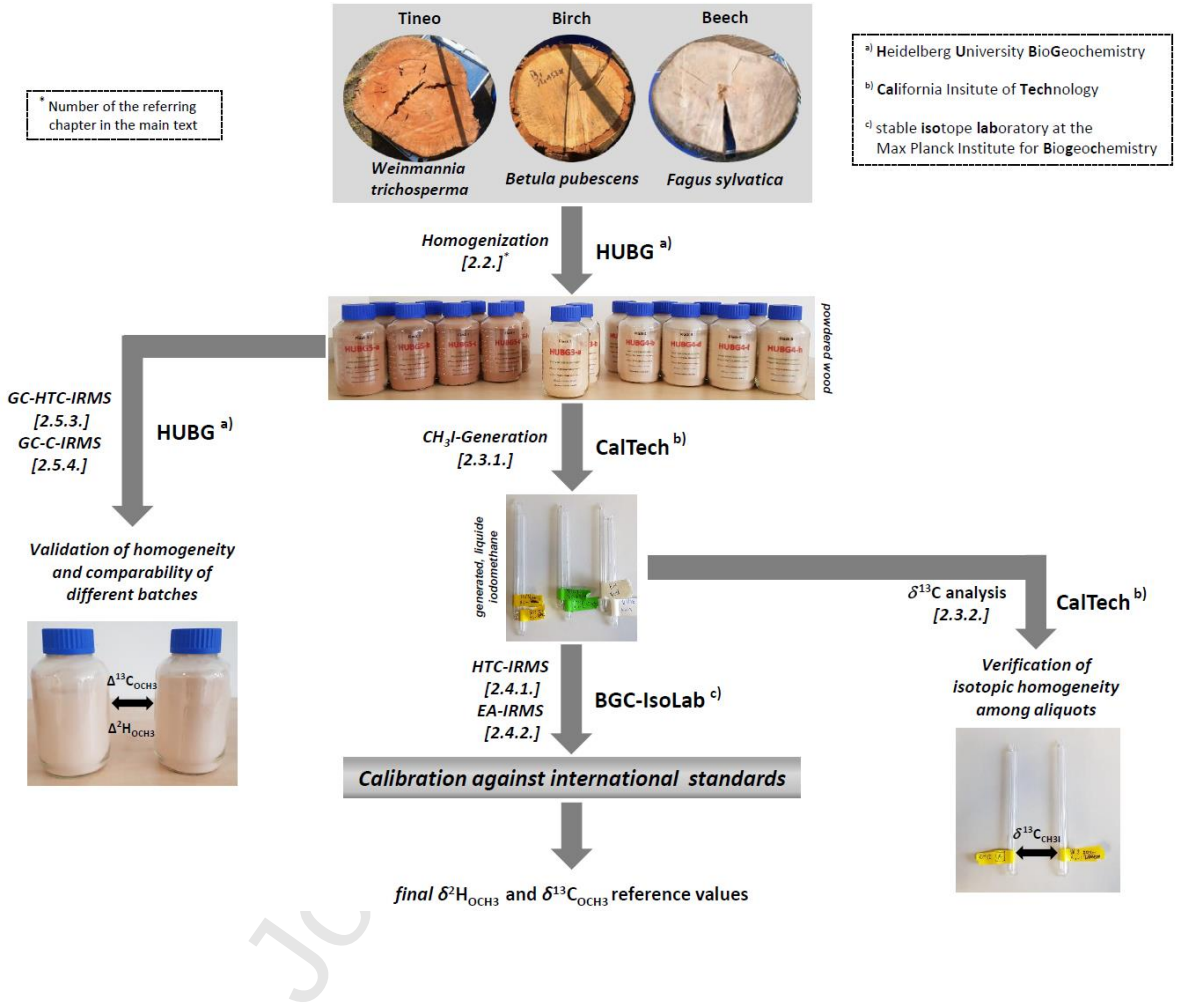
For these reasons, we have recently investigated two methyl sulfate salts (HUBG1 and HUBG2), for their suitability to serve as methoxy reference materials (Greule et al., 2019). These methyl sulfate salts have been demonstrated to be highly suitable to serve as reference materials to normalize $\delta^{13}C_{OCH3}$ values, as they are treated in an identical manner as the

sample and span a relatively wide range of $\delta^{13}C$ values (HUBG1: -50.31 ± 0.16 mUr; HUBG2: +1.60 ± 0.12 mUr) covering most of the natural $\delta^{13}C_{OCH3}$ values of terrestrial plant methoxy groups that have been reported so far (Greule et al., 2019). However, these methyl sulfate salts have a restricted range of $\delta^{2}H_{OCH3}$ values (HUBG1: -144.5 ± 1.2 mUr; HUBG2: -102.0 ± 1.3 mUr) and thus are of limited use for normalizing $\delta^{2}H_{OCH3}$ values of plant methoxy groups, which have $\delta^{2}H_{OCH3}$ values in the range of -150 mUr to -300 mUr (Anhäuser et al., 2015; Greule et al., 2015, 2012; Keppler and Hamilton, 2008; Riechelmann et al., 2017). To properly bracket $\delta^{2}H$ values of plant methoxy groups, additional reference materials, with lower $\delta^{2}H_{OCH3}$ values, are needed. To meet the requirements for stable isotope analysis (Brand et al., 2014; Carter and Barwick, 2011; Schimmelmann et al., 2016; Werner and Brand, 2001), these reference materials must be available in sufficiently large quantities to be applied on a long-term basis and be readily distributable for inter-laboratory comparisons.

To fulfill this need, we have investigated the suitability of three woods from different geographical locations as long-term reference materials for normalization of $\delta^{2}H_{OCH3}$ values. Wood contains relatively high concentrations of methoxy groups in the range of 4-6 % (dry weight basis), primarily originating from lignin. The $\delta^{2}H$ values of these lignin methoxy groups are highly depleted in ^{2}H compared to the $\delta^{2}H$ values of the source water due to a large, uniform apparent biosynthetic isotopic fractionation of -213 ± 17 mUr (Anhäuser et al., 2017b; Keppler et al., 2007). Since the stable hydrogen isotope values of tree source waters generally range from ~0 to -150 mUr, depending on the geographical origin, $\delta^{2}H_{OCH3}$ values of wood lignin can be found in the range of -200 to -350 mUr. Hence, woods from different climatic and geographic regions are ideally suited to serve as reference materials for plant methoxy groups. Wood meets additional requirements for a suitable reference material because it is a non-toxic, solid, stable substance amenable to long-term storage and safe distribution, and can be treated identically to the samples when analyzing stable isotope signatures of methoxy groups.

Here we describe the preparation and analysis of three wood samples (HUBG3, HUBG4 and HUBG5) and make them available as interlaboratory reference materials for $\delta^{2}H_{OCH3}$ and $\delta^{13}C_{OCH3}$ measurements of plant methoxy groups. We detail the procedures (Fig. 1) used to verify the homogenization of these materials, and the techniques used to determine their $\delta^{2}H_{OCH3}$ and $\delta^{13}C_{OCH3}$ values with high confidence.

Figure 1: Illustration of the calibration workflow for the three wood isotopic materials.



2. Material and Methods

2.1. Wood samples

Slices of three tree specimens of different species were collected from three different locations spanning the globe. Details on their origin, mass, and methoxy content are given in Table 1.

2.2. Homogenization of wood samples

Prior to the homogenization of wood samples dried at ambient temperature, the bark of each tree slice was removed. Abundant sawdust from each sample was generated by repeatedly slicing each sample with a bench saw. The sawdust of each sample was collected in a barrel and thoroughly mixed by stirring and shaking. These sawdusts were then reduced to fine powders and further homogenized using a planetary ball mill (Pulverisette 5/4 classic line, Fritsch, Idar-Oberstein, Germany). These powdered and homogenized wood samples were stored in 2L glass flasks under argon atmosphere. Two flasks were prepared for birch wood (HUBG3 a–b) and eight flasks were prepared of beech (HUBG4 a–h) and tineo (HUBG5 a–h) wood samples. Each flask contains about 0.8 to 1.2 kg of pulverized wood.

2.3.1. Preparation and purification of iodomethane from wood samples

Preparation and purification of CH_3I from wood samples was conducted at the California Institute of Technology (Caltech). Pure CH_3I was generated in millimolar quantities by reacting powdered woods with hydriodic acid at reflux in the distillation apparatus depicted in Fig. 2.

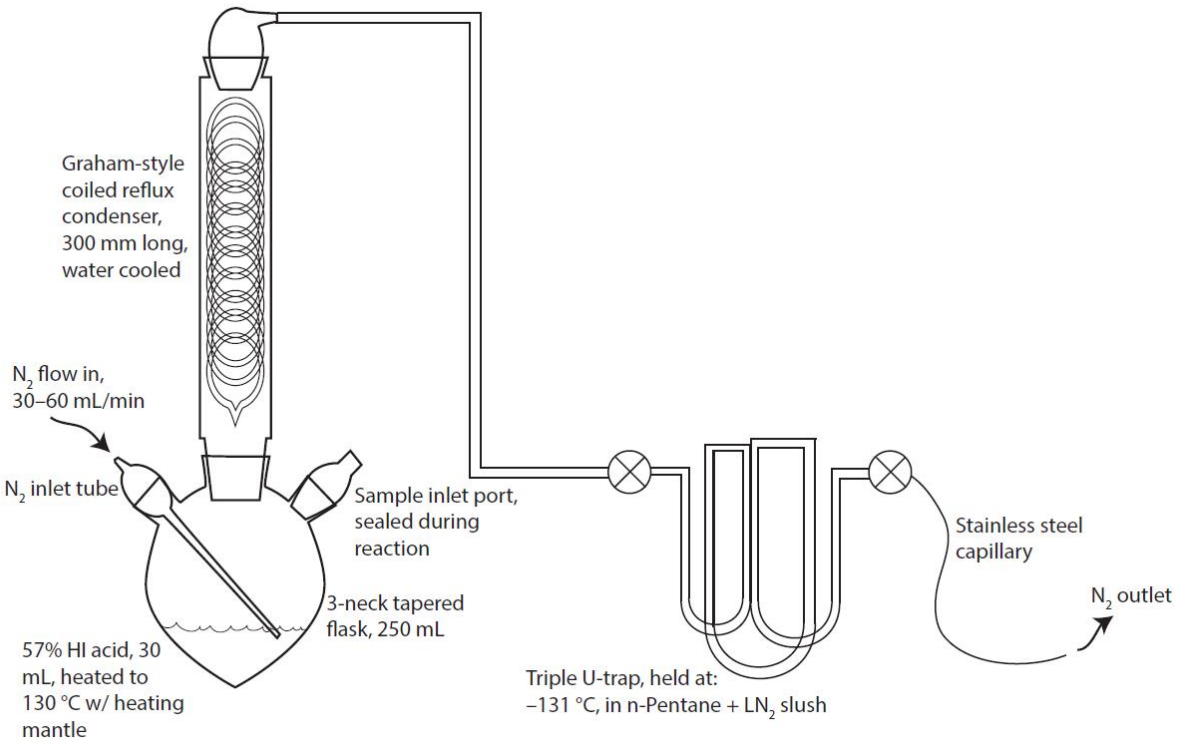


Figure 2: Distillation apparatus used to quantitatively convert wood methoxy groups into

iodomethane (CH_3I). Triple U-trap consists of a single ¼" glass tube blown into three U-bends, sealed with Teflon® valves with Viton® o-rings.

Specifically, 1000 to 2050 mg of powdered wood was added to a tapered 250 mL reaction bulb with 30 mL of 57 wt.% distilled, unstabilized, hydriodic acid in water (99.99% purity, Spectrum Chemical, USA) held at -4 °C. The reaction vessel was attached in series to a Graham-style, water-cooled reflux condenser, glass triple-U trap, and stainless steel outlet capillary, inspired by the apparatus of Krueger and Krueger (1983) designed for a similar purpose. The entire reaction line was gas-tight, with all connections made with either 24/40 ground glass with Dow-Corning® silicone vacuum grease or Cajon Ultra-Torr® vacuum fittings. Immediately after the wood was added, the sample addition port was sealed and the entire apparatus was purged with 99.99% N_2 at 100 mL/min for 5 minutes through a bubbler inlet positioned below the acid level. Then, the flow was reduced to 40 mL/min, and the U-trap was cooled to -131 °C in a liquid N_2 + n-pentane slush to begin trapping evolved volatiles. The hydriodic acid was heated to reflux (130 °C) using a hemispherical heating mantle powered by a Variac® transformer at 50 V to quantitatively convert lignin methoxy groups to CH_3I via the Zeisel reaction (Zeisel, 1885):

$R–O–CH_3 + HI_{(aq)} \rightarrow R–O–H + CH_3I$ (130°C, 90 mins)

The exact temperature at which methoxy groups begin to derivatize is not known. However, since less than 20% of wood methoxy groups derivatize in 15 min at 70 °C (Greule et al., 2008), no significant production of CH_3I likely occurs during the 5 min purge step at room temperature before the acid is heated and the triple U-trap cooled. Evolved CH_3I was continuously entrained in the N_2 flow, stripped of most water and volatile HI on the condenser, and frozen in the triple U-trap held at -131 °C. The reaction was carried out for 1.5 hours to ensure complete conversion of lignin methoxy groups to CH_3I. Preliminary tests and previous work have demonstrated that no further CH_3I is generated after this time (Greule et al., 2008). After 1.5 hours, the N_2 flow was interrupted at the inlet and the triple U-trap was immediately isolated by sealing high-vacuum PTFE valves with Viton® O-rings on either side before the N_2 purge had fully ceased.

Once sealed, the triple U-trap was connected in series to two additional vacuum traps: a water trap held at -77 °C (trap A), and a CH_3I trap held at –196 °C (trap B). The downstream side of trap B was connected to a permanent glass vacuum line pumped by a Hg-diffusion pump. Headspace N_2 was removed by evacuating the triple U-trap across trap A, which was held at -77 °C in slush of 100 % ethanol and CO_2 ice, and trap B, held at –196 °C in liquid N_2. Once all non-condensable gases were removed, the triple U-trap was warmed to 23 °C in a water bath, and its contents were passed in succession across trap A and frozen in trap B over the course of 60 minutes. After 60 mins, trap B was isolated, thawed to room temperature, and CH_3I captured in trap B was passed onto the permanent glass vacuum line by freezing in liquid N_2. This CH_3I was purified of H_2O and CO_2 using the following cryogenic procedures: 1) Trace H_2O was removed by thawing CH_3I in an ethanol–CO_2 ice slush in an isolated vacuum chamber, and then condensing the CH_3I headspace in an adjacent vacuum trap at -196 °C for 30 mins (CH_3I vapor pressure at -77 °C ≈ 78 Pa). This purification was repeated three times until residual water content fell below 1 Pa. 2) CO_2 and similarly-volatile gases were removed by thawing CH_3I at 23 °C in a water bath in an isolated vacuum chamber, freezing CH_3I at -131 °C in a slush of n-pentane + liquid N_2, and evacuating the headspace (mostly CO_2). This purification was repeated five times until headspace pressure at -131 °C reached detection limits (0.13 Pa).

Total CH_3I content was determined by expanding the gas into six connected, evacuated chambers of known volume (total volume: 249 mL) and measuring the pressure by manometry at 25 °C. For each of the three samples, pressure during this expansion never exceeded half of the vapor pressure of CH_3I (71.8 kPa at 25 °C), so the use of the ideal gas law is a reasonable approximation. Iodomethane gases were allowed to isotopically equilibrate among all connected chambers for 15 minutes, after which the valves between the chambers were closed and the contents of individual chambers were quantitatively frozen and sealed into evacuated, pre-combusted borosilicate glass break-seals.

2.3.2. δ^{13}C determination by sealed-tube combustion

To verify that aliquots of CH_3I were isotopically homogenized among vacuum line chambers, the δ^{13}C values of two break-seals from disparate chambers for each of the three standards were determined using standard methods for offline sealed-tube combustion and dual-inlet IRMS. Briefly, 10 to 100 µmol sub-aliquots of each CH_3I aliquot were sealed in evacuated

quartz break-seals with 40 to 200 mg of pre-combusted cupric oxide (needles, 99.9% purity, Sigma Aldrich #310433) and 40 mg of pre-combusted silver foil (3.5 x 5 mm pressed silver capsules, Costech® #041066). These tubes were combusted for 4 hrs at 825 °C, wherein CH_3I was quantitatively converted to CO_2, AgI, and H_2O. Product CO_2 aliquots were cleaned of H_2O on a glass vacuum line using standard cryogenic purification techniques and sealed into clean borosilicate break-seals. These were measured for $\delta^{13}C$ and $\delta^{18}O$ values on a Thermo Delta V IRMS in dual-inlet mode against a CO_2 tank of known isotopic composition (Oztech). These 'raw' $\delta^{13}C$ and $\delta^{18}O$ values (i.e., not scale-calibrated) were merely used to assess homogeneity among aliquots of CH_3I, not for absolute $\delta^{13}C$ calibration.

2.4. Isotopic calibration of iodomethane generated from methoxy groups

2.4.1. Hydrogen isotopic analyses by HTC-IRMS

The stable hydrogen and carbon isotopic ratios of the purified CH_3I from the sealed vials prepared at Caltech (see above) were analyzed at the Max Planck Institute for Biogeochemistry (BGC-IsoLab). The hydrogen isotopic values were determined by High-Temperature Conversion-Isotope Ratio Mass Spectrometry (HTC-IRMS). We refer the reader to Gehre et al., 2004 regarding method details. Briefly, the sample break seal tubes were cooled in an ice bath to 0 °C. Subsequently samples were quickly transferred into GC-vials containing glass inserts for microvolumes. For each measurement, 3 µl of CH_3I were manually injected into the HTC oven. The conversion to H_2 gas was achieved with a high-temperature furnace (Hekatech, Wegberg, Germany) held at 1430 °C, that was equipped with a glassy carbon tube inside a silicon carbide tube (tube-in-tube design) with a reverse feed He carrier gas flow of ~ 80 ml/min. Gehre et al. (2017, 2015) and Nair et al. (2015) have demonstrated the importance of filling the HTC reactor with chromium when analyzing the δ^2H values of heteroatom-containing compounds (N, S, F, Cl, Br, I). Chromium traps the heteroatoms and thus facilitates a quantitative conversion of analyte to H_2 gas, and subsequent transport to the IRMS (Gehre et al., 2015). Consequently, the analyses of the CH_3I and associated standards were done using a glassy carbon tube filled with elemental chromium and glassy carbon chips. The HTC-furnace was coupled to a Delta^{plus}XL IRMS via a ConFlo III open-split interface (both Thermo Fisher Scientific). For normalization of data and applied reference materials we would like to refer to section 3.2.1. "Calibration of δ^2H_{OCH3} values".

2.4.2. Carbon isotopic analyses by EA-IRMS

The carbon isotopic composition of CH_3I generated from methoxy groups were measured on a Thermo Fisher Delta^{plus} IRMS coupled to an Elemental analyser (EA; Carlo Erba 1100 CE analyser; Thermo Fisher Scientific, Rodano, Italy) via a ConFlo III open-split interface. The measurement procedure has been described previously (Brooks et al., 2003; Werner et al., 1999; Werner and Brand, 2001). In brief, sample-containing break seals were cooled in an ice bath and then samples were transferred into GC-vials containing glass inserts. A drop of sample was manually placed in a tin capsule, sealed and introduced into the EA via the autosampler. The samples were combusted at 1020 °C in the oxidation oven, and then reduced at 650 °C. Water was removed with a Nafion^{TM} and magnesium perchlorate trap before the sample gas was introduced into the gas chromatograph held at 80 °C, containing a HaysepQ-filled 2m packed column (IVA-Analysetechnik, Meerbusch, Germany). The He-carrier gas flow was 110 ml/min. For normalization of data and applied reference materials we would like to refer to section 3.2.2.

2.5. Stable isotope analysis of wood methoxy groups using GC-IRMS

2.5.1. Generation of iodomethane from wood, lignin, vanillin and pectin

Continuous flow GC-HTC-IRMS measurements were conducted at Heidelberg University, Institute of Earth Sciences, Heidelberg, Germany (HU). Analysis of δ^2H_{OCH3} values of CH_3I, released upon treatment of the samples with HI (hydriodic acid, 57 wt.% aqueous solution, Acros (Thermo Fisher Scientific), Geel, Belgium) was carried out using the method described by Greule et al., 2008. Hydriodic acid (0.25 ml) was added to the samples (stable carbon analysis: 5 mg, stable hydrogen analysis: 10mg) in a crimp-top glass vial (1.5 ml; IVA Analysentechnik, Meerbusch, Germany). The vials were sealed with crimp caps containing PTFE-lined butyl rubber septa (thickness 0.9 mm) and incubated for 30 min at 130 °C. After heating, the samples were allowed to equilibrate at room temperature (22 ± 0.5 °C) for at least 30 min before an aliquot of the headspace (80 – 90 µl) was directly injected into the analytical system using a gas-tight syringe (100 µl, SGE Analytical Science).

2.5.2. Hydrogen stable isotope analysis using GC-HTC-IRMS

δ^2H values of the CH_3I were measured using an HP 6890N gas chromatograph (Agilent, Santa Clara, USA) equipped with an auto sampler A200S (CTC Analytics, Zwingen, Switzerland), coupled to a Delta^{PLUS}XL isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a thermo conversion reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., reactor temperature 1450 °C] and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany).

The gas chromatograph (GC) was fitted with a Zebron ZB-5MS capillary column (Phenomenex, Torrance, USA) (30m x 0.25mm i.d., d_f 1µm) and the following conditions were employed: split injection (4:1), initial oven temperature at 30 °C for 3.8 min, ramp at 30 °C/min to 100 °C. Helium was used as carrier gas at a flow of 0.6 mL/min constant flow.

A tank of high purity hydrogen gas (Alphagaz^{TM} 2 H_2, Air Liquide, Düsseldorf, Germany) was used as the monitoring gas. The H_3^+ factor ranged from 2.18 to 2.35 ppm/nA. δ^2H values of materials presented in Table 3 and 4 were normalized by a linear normalization algorithm (Carter and Barwick, 2011) using reference materials presented in Table 2.

2.5.3. Carbon stable isotope analysis using GC-C-IRMS

δ^{13}C values of CH_3I were analyzed using the same analytical system described for hydrogen measurements, except that the GC and MS were coupled by an oxidation reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., with Cu/Ni/Pt wires inside (activated by oxygen), reactor temperature 960°C] and different GC conditions were employed: split injection (10:1), initial oven temperature at 40 °C for 3.8 min, ramp at 30 °C/min to 100 °C. Helium was used as carrier gas at a flow of 1.8 mL/min constant flow.

A tank of high purity carbon dioxide (grade 4.8) (Kohlendioxid ISO-TOP, Air Liquide, Düsseldorf, Germany) was used as the monitoring gas. δ^{13}C values were normalized by a linear normalization algorithm (Carter and Barwick, 2011) using the reference materials HUBG1 and HUBG2 described in Table 2.

3. Results and discussion

3.1. Quantitative conversion of methoxy groups from the three wood samples to iodomethane

First, methoxy groups from subsamples of the three homogenized wood slices (for detailed description please see Method section 2.3.1.) with measured methoxy content of 4.7, 5.4 and 5.1% for birch, beech and tineo, respectively were quantitatively converted to CH_3I. Four aliquots of each wood sample were generated and stored in borosilicate glass break-seals. To verify that aliquots of CH_3I were isotopically homogenized among vacuum line chambers, the $\delta^{13}C$ values of two break-seals from disparate chambers for each of the three standards were determined using standard methods for offline sealed-tube combustion and dual-inlet IRMS (see section 2.3.2.). Relative deviations in the $\delta^{13}C$ values of different aliquots of the same wood sample were comparable to typical standard deviations of $\delta^{13}C$ values from separate sealed-tube combustions of homogeneous materials (~0.15mUr), suggesting that CH_3I of sub-aliquots of each wood sample was not significantly fractionated in ^{13}C content during apportionment. The absolute $\delta^{13}C$ values from the offline sealed-tube combustion/dual-inlet IRMS workflow are not used for the calibration exercise because this dual-inlet system was not calibrated to the VPDB scale using two scale anchors.

3.2. Calibration of iodomethane generated from the three wood species

3.2.1. Calibration of δ^2H_{OCH3} values

The δ^2H_{OCH3} values for hydrogen of HUBG3, HUBG4 and HUBG5 measured by HTC-IRMS are -272.9 ± 1.5 mUr (n=11), -239.1 ± 1.4 mUr (n=10) and -191.7 ± 0.8 mUr (n=9), respectively (cf. Table 2). The errors given represent the combined uncertainties which were calculated by error propagation containing contributions from the weighted standard error of the scaling standards and the precision of replicate measurements and the algorithms applied to correct and normalize the data (Carter and Barwick, 2011). The complete propagated uncertainty of the wood standards $U_{HUBG3/4/5}$ is calculated as:

$$U_{HUBG3/4/5} = \sqrt{u_{uss}^2 + u_{HUBG3/4/5}^2}$$

where u_{uss} indicates the weighted analytical uncertainty of the scaling standards and $u_{HUBG3/4/5}$ indicates the measurement uncertainty of the CH_3I generated from the three wood standards HUBG3, HUBG4 and HUBG5. All uncertainties are multiplied by the Student's factor (t) at a 90 % confidence limit to account for the limited number of analyses.

The CH_3I aliquots measured at BGC-IsoLab were normalized to the VSMOW/ isotopic δ scale using a two-point calibration against VSMOW2 (0 ± 0.3 mUr) and SLAP2 (-427.5 ± 0.3 mUr). The in-house water standard WWW-J1 (Willi Working Water; -66.45 ± 1 mUr) was interspersed throughout the samples and used as a quality control.

3.2.2. Calibration of $\delta^{13}C_{OCH3}$ values

$\delta^{13}C_{OCH3\text{-}VPDB(LSVEC)}$ values for carbon of HUBG3, HUBG4 and HUBG5 measured by EA-IRMS are -29.40 ± 0.13 mUr (n=9), -30.17 ± 0.13 mUr (n=8) and -29.77 ± 0.13 mUr (n=8), respectively (cf. Table 2). The errors presented were calculated in the same way as those provided for the stable hydrogen isotope values. They represent the combined uncertainties containing the same contributions as mentioned for the stable hydrogen measurements above. The CH_3I samples generated from the three wood standards measured at the BGC-IsoLab by EA-IRMS were calibrated to the VPDB scale using the reference material IAEA-603 (+2.46 ± 0.01 mUr) and an in-house standard as the second scale anchor (Acetanilide; -30.06 ± 0.1 mUr), which was calibrated against the two international secondary standards NBS 22 (-30.03 ± 0.04 mUr) and LSVEC (-46.6 ± 0.2 mUr). As of June 2018, IUPAC no longer recommends the use of LSVEC as a scaling standard for the VPDB scale (Press release; http://iupac.org/standard-atomic-weights-of-14-chemical-elements-revised). LSVEC has previously been shown to be an inadequate $\delta^{13}C$ isotopic standard (Assonov et al., 2015; Qi et al., 2016b). Over time, LSVEC carbon atoms exchange with atmospheric CO_2 carbon atoms. This causes a gradual contamination and ^{13}C-isotopic fractionation of LSVEC. The BGC-Isolab is currently involved in an effort to produce a replacement standard for LSVEC. The envisaged replacement is USGS44, a Merck high purity $CaCO_3$ with a preliminary value of -42.15 ± 0.05 mUr, available at https://isotopes.usgs.gov. It is possible that USGS44 will replace LSVEC in the future. Therefore, BGC-IsoLab also analyzed HUBG 3-5 using USGS44 as the second scale anchor. We note that the provided USGS44 value is a preliminary value and may change slightly upon its final publication. However, if necessary,

users of HUBG 3-5 can easily recalculate the values of HUBG 3-5. The preliminary $\delta^{13}C_{OCH3\text{-}VPDB(USGS44)}$ values of HUBG3, HUBG4 and HUBG5, calibrated against IAEA-603 and scaled to USGS44 (-42.15 ± 0.05 mUr) are -29.30 ± 0.10 mUr (n=9), -30.07 ± 0.10 mUr (n=8) and -29.65 ± 0.11 mUr (n=8), respectively (cf. Table 2). Please note that the results of HUBG 3-5 obtained from EA-IRMS were also compared with measurements made by GC-C-IRMS applying the method as described in section 2.5. and using HUBG1 and HUBG2 as reference materials for normalization of $\delta^{13}C_{OCH3}$ values. We found good agreement of $\delta^{13}C_{OCH3}$ values for HUBG3-4. However, we also noted some inconsistencies for HUBG5 (tineo wood) when comparing the results of the two applied methods. We have currently no obvious explanation for these deviations. For that reason, we do currently not suggest HUBG5 for usage as a reference material to normalize $\delta^{13}C$ values of methoxy groups.

3.3. Comparability and homogeneity of powdered wood batches

To ensure the homogeneity of different batches of each wood sample (HUBG3: 2 flasks, HUBG4/5: 8 flasks each, see Table 1) four to five sub-samples from each flask were analyzed for both $\delta^{13}C_{OCH3}$ and $\delta^{2}H_{OCH3}$ values by continuous flow GC-C/HTC-IRMS according to methods established by Greule et al. (2008 and 2009). Deviations in isotopic composition from sub-samples of all flasks from each series (wood sample) relative to the mean value of all measurements (raw data) of one series are shown in Fig. 3a ($\delta^{13}C_{OCH3}$) and Fig. 3b ($\delta^{2}H_{OCH3}$). The average isotope differences within subsamples (filled grey circles) of one flask ($\delta^{13}C$: n=5; $\delta^{2}H$: n=4) were 0.07 mUr (range 0.02 to 0.19 mUr) and 0.96 mUr (range 0.17 to 2.91 mUr) for $\Delta\delta^{13}C_{OCH3}$ and $\Delta\delta^{2}H_{OCH3}$ values, respectively.

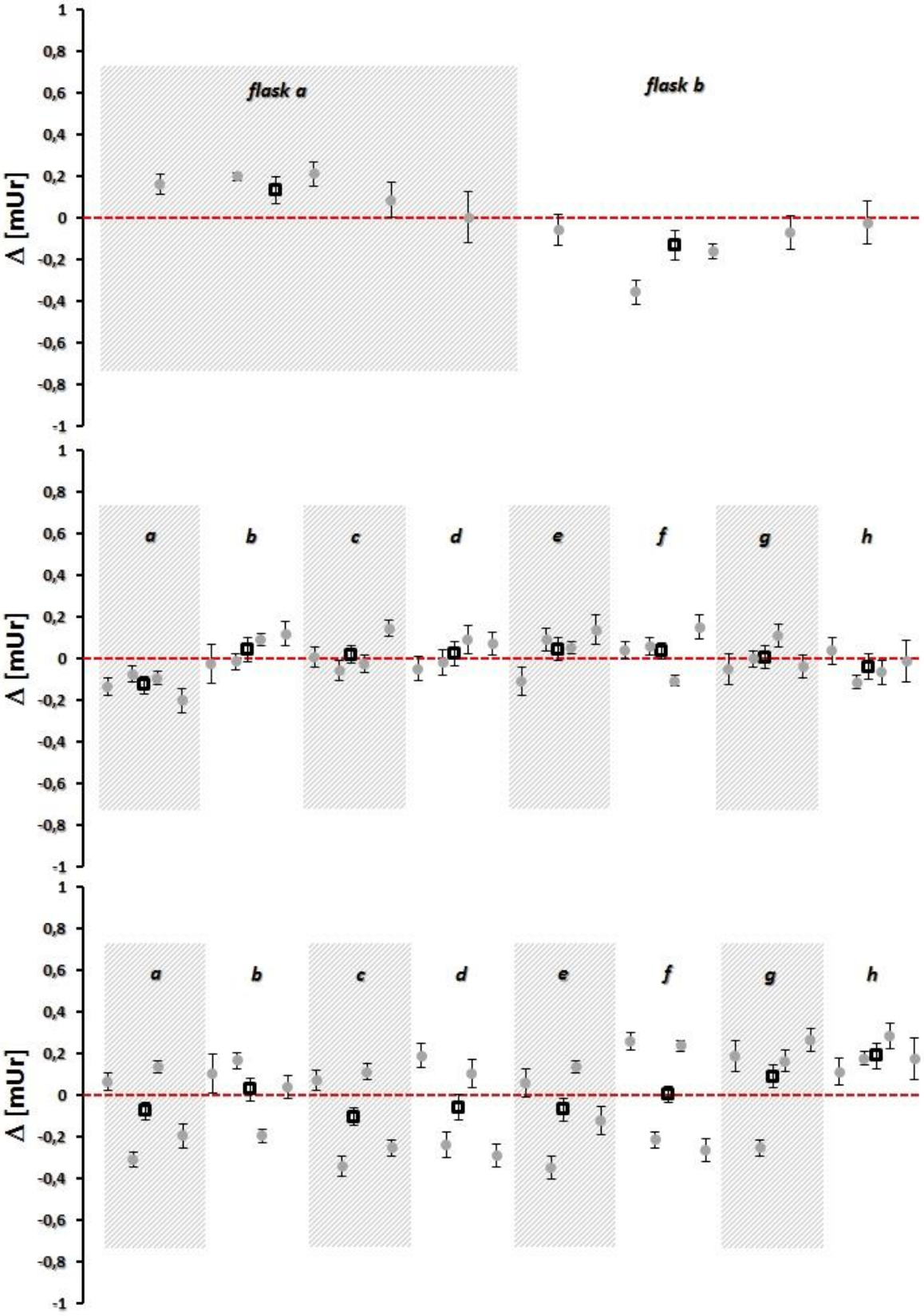


Figure 3a: $\delta^{13}C$ isotope differences from sub-samples of all flasks from one batch series (upper panel: HUBG3 (birch), middle panel: HUBG4 (beech), lower panel: HUBG5 (tineo)) relative to the mean value of all measurements of one batch

series. Black, open squares indicate the mean value of all sub-samples from one flask. Error bars show the standard deviations from the replicate measurements of each sub-sample (1σ, n=5).

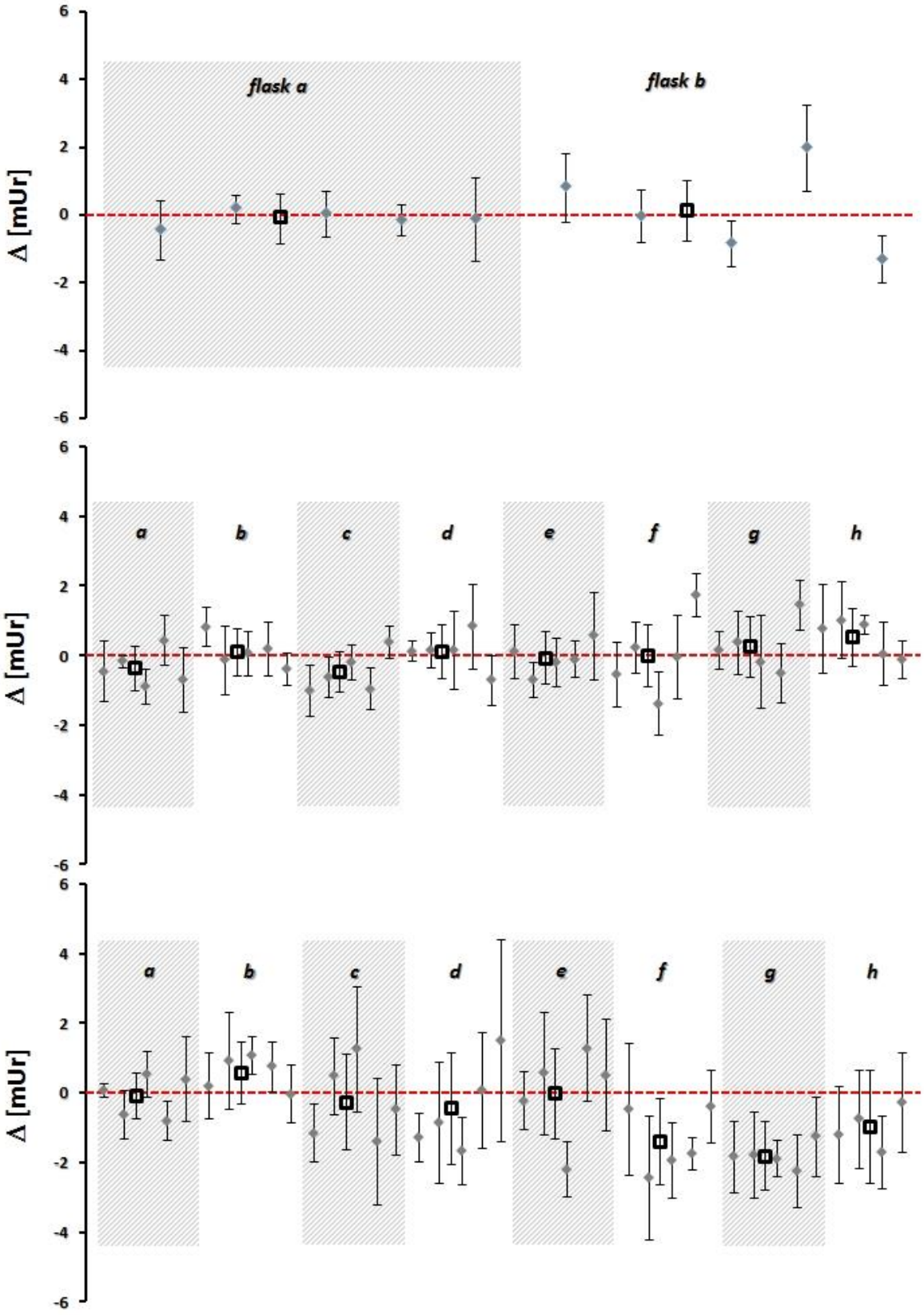


Figure 3b: δ^2H isotope differences from sub-samples of all flasks from one batch series (upper panel: HUBG3 (birch), middle panel: HUBG4 (beech), lower panel: HUBG5 (tineo)) relative to the mean value of all measurements of one batch

series. Black, open squares indicate the mean value of all sub-samples from one flask. Error bars show the standard deviations from the replicate measurements of each sub-sample (1σ, n=4).

When comparing differences in isotopic composition of the mean values (black squares) of each flask within one wood series/species (upper, middle and lower panel) we found maximum $\Delta\delta^{13}C_{OCH3}$ values of 0.19 mUr (0.13, 0.13 and 0.19 mUr for HUBG3, HUBG4 and HUBG5) and $\Delta\delta^{2}H_{OCH3}$ values of 1.82 mUr (0.11, 0.51 and 1.82 mUr for HUBG3, HUBG4 and HUBG5). All measured differences within sub-samples of one flask and the whole batch series (except one sub-samples from flask HUBG3-b and one from HUBG5-e) are comparable to the internal precision for $\delta^{13}C$ and $\delta^{2}H$ measurements (< 0.3 mUr and 3 mUr, respectively) which the manufacturer states that this IRMS instrument should attain. In summary, we suggest that all flasks of one batch series containing the same wood are homogeneous regarding $\delta^{13}C_{OCH3}$ and $\delta^{2}H_{OCH3}$ values and thus are ideally suited for long-term usage, inter-laboratory comparison, and thus traceability to reference materials.

3.4. Discussion

The three wood reference materials HUBG3-5 calibrated within this work span a range of $\delta^{2}H_{OCH3}$ values from -191 to -273 mUr. In combination with two recently investigated methyl sulfate salts HUBG1 and HUBG2 (Greule et al., 2019), the isotopic range of solid methoxy reference materials cover $\delta^{2}H$ values from roughly -100 to -300 mUr (cf. Table 2). For stable carbon isotope measurements, the two wood reference materials HUBG3-4 only provide a narrow range of ~-29 mUr to -30 mUr. However, in combination with HUBG1 and HUBG2 (Greule et al., 2019), the full range of $\delta^{13}C_{OCH3}$ covered by the available reference materials values is from +2 mUr to -51 mUr and thus convers most of the natural variation in $\delta^{13}C$ values of terrestrial plant methoxy groups reported so far. Moreover, the two wood reference materials HUBG3-4 are highly suitable for standardizing $\delta^{13}C$ measurements of wood methoxy groups as their $\delta^{13}C_{OCH3}$ values (cf. Table 2) are in the same range as most of the wood methoxy group values that have been measured previously (~ -20 to -30 mUr) (Gori et al., 2013; Mischel et al., 2015; Riechelmann et al., 2016).

Up to now, most of the reported stable isotope values of methoxy groups analyzed by IRMS were normalized to the respective δ-scale using liquid CH_3I as the reference material. As

discussed above, this practice has some disadvantages. Therefore, we suggest replacing liquid CH_3I by the solid reference materials set HUBG1-5 and principal working standards for normalizing δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values to the respective δ-scale.

A major advantage of using solid reference materials is the superior suitability for long term storage and transport when used for inter-laboratory comparisons, whereas volatile CH_3I standards may evaporate with time and require careful handling during distribution. Another disadvantage of using CH_3I as the reference material is that δ^2H and $\delta^{13}C$ values of commercially available CH_3I have been shown to cover only a narrow range (δ^2H_{CH3I}: -179 to -66 mUr; $\delta^{13}C_{CH3I}$: -70 to -46 mUr (Feakins et al., 2013b; Greule et al., 2019; Keppler et al., 2007; https://arndt.schimmelmann.us/files/alphabetical list of all reference materials.pdf)). This narrow range is insufficient to bracket samples and monitor issues of scale compression. Thus, existing published measurements of δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values might be compromised due to a violation of the principle of identical treatment and a lack of accounting for scale compression. We suggest that most results of plant methoxy groups which have been published so far may need to be corrected when they considerably diverge from the isotopic range covered by the used reference materials. This might be particularly necessary for existing analyses of δ^2H and $\delta^{13}C$ values of wood methoxy groups because these values are so distinct from CH_3I standards (Anhäuser et al., 2017b, 2017a; Gori et al., 2013; Mischel et al., 2015; Riechelmann et al., 2017, 2016).

Applying the new set of solid methoxy reference materials, we have re-analysed four methoxy group containing compounds which have been measured previously using iodomethane as the reference material (Greule et al., 2009, 2008). Table 3 compares the data measured previously with the new results obtained using HUBG2 and HUBG3 as reference materials for normalization of the isotopic values to the respective δ-scale. The results show that there are relatively large deviations ($\Delta\delta^2H$ ranging from 11 mUr to 27 mUr, and $\Delta\delta^{13}C$ around 3 mUr) between previous and current measurements. It is also obvious that deviations of δ^2H_{OCH3} values are highest for the most negative δ^2H_{OCH3} values (-250 to -300 mUr). This might be easily explained because normalization of the previous measurements was done by using only one CH_3I working reference standard which was initially calibrated with δ^2H and $\delta^{13}C$ values of -179.0 ± 2.9 mUr (n=15, 1σ) and -69.27 ± 0.05 mUr (n=15, 1σ) (Greule et al., 2009, 2008). Later, the CH_3I reference material was re-calibrated (Anhäuser et al., 2017b) with δ^2H and $\delta^{13}C$ values of -173.0 ± 1.5 mUr (n = 9, 1σ) and -66.2 ± 0.13 mUr (*n*=8, 1σ), respectively. Thus it becomes clear that for both δ^2H and $\delta^{13}C$ values of the previous applied CH_3I

reference material there is a large gap between naturally occurring δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values of wood samples and the previously used CH_3I reference material.

Also applying the new set of methoxy reference materials we additionally analyzed the δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values as well as the methoxy contents of three wood standards (USGS54-56) which were recently introduced as reference materials for δ^2H, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$ bulk measurements (Qi et al., 2016a).

4. Conclusion

We suggest that the investigated three wood reference materials HUBG3-5 are highly suited for normalization of δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values of plant origin. Moreover, they are ideal for long-term usage and inter-laboratory comparison. The δ^2H_{OCH3} values of the three wood samples span a relatively wide range (~-280 to -190 mUr) suitable for normalization of δ^2H_{OCH3} values of most plant samples that have been reported so far. $\delta^{13}C_{OCH3}$ values of the two wood samples (HUBG3-4) are restricted in range (-30 to -29 mUr), but are typical for $\delta^{13}C_{OCH3}$ values reported for wood methoxy groups. However, together with the two recently reported methyl sulfate salts HUBG1 and HUBG2 (Greule et al., 2019) they form a complete set of solid methoxy reference materials that covers δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values from -280 to -100 mUr and -50 to +2 mUr, respectively (Table 2). This sample set covers almost the full range of plant methoxy groups reported so far.

The relatively large deviations reported for the re-analysis of the four methoxy group containing compounds (Table 3) demonstrates the importance of adherence to the IT principle and the use of two-point calibrations when applying stable isotope analysis of plant methoxy groups. Based on our results, we suggest that most of δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values of plant methoxy groups reported so far might need to be corrected. For future measurements we recommend replacing liquid CH_3I with the solid reference materials HUBG1-5 for normalizing δ^2H_{OCH3} and $\delta^{13}C_{OCH3}$ values to the respective δ-scale. Finally, we would like to advice all potential users that for reliable measurements of the stable isotope composition of methoxy groups it is of crucial importance to comply with the description of the preparation of CH_3I as outlined in this and previous publications (Greule et al. 2008 & 2009) and adhere to the principle of identical treatment of samples and solid reference materials.

5. Source for methoxy reference material

HUBG3, HUBG4, and HUBG5 methoxy wood isotopic reference materials as well as HUBG1 and HUBG2 methyl sulfate salts are available upon request from:

University Heidelberg, Institute of Earth Sciences, Biogeochemistry Group, INF 236, 69120 Heidelberg, Germany

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Table 1: Detailed information on the wood used as isotopic reference materials.

ID	Tree common name	Species	Total amount [a] [kg]	Geographic origin	Altitude [m]	Number of flasks	Methoxy content [b] [%]
HUBG3 a-b	Birch	*Betula pubescens*	1.8 kg	59°12'36.35"N 29°45'44.19"E	106 m	2	4.71 ± 0.09 %
HUBG4 a-h	Beech	*Fagus sylvatica*	9.2 kg	49°22'48.53"N 8°46'43.39"E	257 m	8	5.40 ± 0.08 %
HUBG5 a-h	Tineo	*Weinmannia trichosperma*	7.2 kg	40°15'59.77"S 72°10'39.81"W	100 m	8	5.09 ± 0.07 %

[a] amount of powdered wood after preparation/homogenization

[b] analyzed by GC-FID according to Li et al., 2012

Table 2: Calibrated stable hydrogen and carbon isotope values of reference materials available from Heidelberg University.

ID	Compound	$\delta^2H_{OCH3\text{-}VSMOW}$ [mUr]		$\delta^{13}C_{OCH3\text{-}VPDB}$ [b)] [mUr]		$\delta^{13}C_{OCH3\text{-}VPDB}$ [c)] [mUr]	
HUBG1 [a)]	Sodium methyl sulfate	-144.5 ± 1.2	*n = 30*	-50.31 ± 0.16	*n = 14*	-50.17 ± 0.08	*n = 14*
HUBG2 [a)]	Potassium methyl sulfate	-102.0 ± 1.3	*n = 32*	+1.60 ± 0.12	*n = 16*	+1.60 ± 0.05	*n = 16*
HUBG3	Birch wood	-272.9 ± 1.5	*n = 11*	-29.40 ± 0.13	*n = 9*	-29.30 ± 0.10	*n = 9*
HUBG4	Beech wood	-239.1 ± 1.4	*n =10*	-30.17 ± 0.13	*n =8*	-30.07 ± 0.10	*n =8*
HUBG5	Tineo wood	-191.7 ± 0.8	*n = 9*	-[d]		-[d]	

[a)] (Greule et al., 2019)

[b)] values calibrated against IAEA-603 and scaled to LSVEC (refer to section 3.2.2.)

[c)] values calibrated against IAEA-603 and scaled to USGS44 (refer to section 3.2.2.)

[d)] value not provided due to inconsistencies between measurements made by GC-IRMS and EA-IRMS

Table 3: Comparison of four methoxy group containing compounds normalized against iodomethane (previous) and HUBG2/3 (δ^2H values) and HUBG1/2 (δ^{13}C values) (new measurements). δ-values were obtained by GC-C/HTC-IRMS analysis after conversion of the methoxy groups to CH_3I.

		Vanillin		Lignin		Wood		Pectin	
$\delta^2H_{OCH3\text{-}VSMOW}$ [mUr]	***previous measurements*** [a)]	-111.2 ± 1.1	*n = 10*	-253.0 ± 0.7	*n = 10*	-268.5 ± 1.6	*n = 10*	-289.6 ± 1.5	*n = 10*
	new measurements [b)]	-100.6 ± 3.4	*n = 15*	-235.7 ± 2.5	*n = 15*	-242.0 ± 2.5	*n = 14*	-269.6 ± 2.4	*n = 15*
	Δ (previous – new)	10.6		17.3		26.5		20.0	
$\delta^{13}C_{OCH3\text{-}VPDB}$ [mUr]	***previous measurements*** [c)]	-41.73 ± 0.10	*n = 10*	-20.82 ± 0.08	*n = 10*	-24.84 ± 0.13	*n = 10*	-36.33 ± 0.09	*n = 10*
	new measurements [b)]	-44.66 ± 0.37	*n = 15*	-23.83 ± 0.29	*n = 15*	-27.99 ± 0.31	*n = 15*	-39.91 ± 0.28	*n = 15*
	Δ (previous – new)	2.93		3.01		3.15		3.58	

[a)] Greule et al., 2008

[b)] this study

[c)] Greule et al., 2009

Table 4: Methoxy group analysis of the three stable isotope bulk reference standards USGS54-56 normalized against HUBG2/3 (δ^2H values) and HUBG1/2 (δ^{13}C values). δ-values were obtained by GC-C/HTC-IRMS analysis after conversion of the methoxy groups to CH_3I.

ID [a]	Tree common name	$\delta^2H_{OCH3\text{-}VSMOW}$ [mUr]		$\delta^{13}C_{OCH3\text{-}VPDB}$ [mUr]		Methoxy content [b] [%]	
USGS54	Canadian Lodgepole pine	-319.1 ± 3.4	*n = 15*	-25.63 ± 0.34	*n = 15*	3.97 ± 0.08	*n = 10*
USGS55	Mexican ziricote	-210.0 ± 2.9	*n =15*	-32.66 ± 0.32	*n =15*	3.98 ± 0.10	*n =10*
USGS56	South African red ivorywood	-177.6 ± 2.8	*n = 15*	-27.97 ± 0.32	*n = 15*	4.20 ± 0.06	*n = 10*

[a] for more details and δ^2H, δ^{18}O, δ^{13}C, and δ^{15}N bulk values refer to (Qi et al., 2016a)

[b] analyzed by GC-FID according to Li et al., 2012

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: